Focus Strategies with ZEN2





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ZEN Focus Strategies



- Focus Maintenance
 - Software Autofocus (SWAF)
 - Hardware: Definite focus (DF)
- Focus Strategies
 - Focus Strategy window
 - Only Autofocus
 - Only Definite Focus
 - A combination of both
- When to choose which strategy?
 - Different options with examples
 - Local versus Global Support Points

Focus Maintenance

Software Autofocus





- Find Focus runs Autofocus
- Based on parameters set in Software Autofocus window



- **Mode**: Contrast for Widefield, Intensity for Confocal system (Auto will define best option based on system configuration)
- **Quality**: a more optimal algorithm for weaker signals is used (Choose Low Signal for sparse and/or weak signal)
- **Search**: Smart will stop z-movement on the first occurrence of a sharp object, Full will use the complete search range and then return
- **Sampling**: step-size, based on the depth of field of the objective. Typically Default is ok, fine will require 2x more steps (=potentially 2x more bleaching)
 - **Range**: Relative Range is Default, if Automatic is switched off, one can define a larger depth to search. With Fixed Range, a specific minimum & maximum has to be defined (similar to z-stack First/Last).

Focus Maintenance Software Autofocus



Autofocus ROI

Spot Meter / Focus ROI

- Spot Meter / Focus ROI:
 - Mark small region of interest on image for focus maintenance
 - Particularly useful with fiducials (e.g. fluorescent beads)
 - When enabled, a red square will appear in the Live window which can be resized & repositioned





Focus Maintenance Software Autofocus – Widefield

ZEISS



Focus Maintenance

Software Autofocus – Confocal





Focus Maintenance Hardware Autofocus – Definite Focus 2



- Many new options introduced with DF.2 and ZEN2.3 or higher
- Searches for reflection of IR laser on coverslip → no interference with sample, thus no bleaching or phototoxicity



Focus Maintenance Hardware Autofocus – Definite Focus 2



- Find Surface: will detect coverslip based on reflection of IR light
- **Store Focus**: after finding coverslip and focusing (manually or with SWAF), stores the offset (distance) between coverslip and sample
- Lock Focus: will keep DF.2 AND offset active while screening over sample (=continuously maintain focus)
- **Recall Focus**: with Lock Focus inactive, or during an experiment, the previously defined offset is applied once.
- **Period**: DF.2 can be used continuously, set here the interval. 0s is continuously, often 30s or even 1min is sufficient



Focus Maintenance Hardware Autofocus – Definite Focus 2



In menu: Tools | Options | Acquisition | Focus Strategy



• Enabling 'Show Definite Focus Setting "Resolution and Speed" shows the DF accuracy parameter in the Focus Strategy window when using DF



- Exact: very accurate, but slower
- Balanced: good accuracy and speed
- Fast: faster focusing, but possibly less accurate

Focus Strategies Focus Strategy window



- For SWAF, a Reference channel is required
 - Select a channel in Channels or Focus Strategy window & click on Set as Reference



- Choose a channel that can handle some bleaching, or if possible, a transmitted light image
- Beware of dirt on coverslip or sample, it may influence the focus result as it is generally very bright
- Consider the use of fluorescent beads in a channel not used for the experiment (e.g., when imaging BFP, GFP and DsRed, one could use Far-red stained beads for SWAF)

Tiles, Regions & Positions





Positions = collection of single Tiles

Example: screening of cell culture



Region = collection of multiple Tiles,

linked and often overlapping to be able to stitch & fuse into one large image (=extending field of view)

Example: imaging of 1 zebrafish, complete at high magnification



Regions

Combination of Positions & Region: multiple regions, with each a different position.

Example: imaging of multiple zebrafish embryos, complete at high magnification

Focus Strategies Software Autofocus only







- Without Tiles enabled, Time lapse SWAF runs before every nth acquisition
- With Tile enabled, two options:
 - Before every time point in center of region
 - Before every time point on first tile of region

Focus Strategies Software Autofocus only



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- When using multiple Positions or Regions, time-lapse runs SWAF on every Region.
- To speed up acquisition, select Region/Position and choose to skip positions or regions.
- Setting it to "Repeat Every 1 R/P" is similar to selecting "Check SWAF every time-point"

 For large Region(s), one can benefit from checking SWAF every nth Tile to cover for sample unevenness

Focus Strategies Definite Focus only





- Definite Focus has similar options as SWAF
- One extra option: Enable Periodic Stabilization
 - For time-lapse with long interval (>5min), drift may be too high resulting in DF not finding the reflection anymore when only checking every time-point or position
 - With Periodic Stabilization enabled, DF will maintain focus also during the interval, e.g. every 10s

Focus Strategies Combined SWAF and DF



- A combination offers more options and often higher accuracy & speed
- **SWAF as reference for DF**: useful e.g. when SWAF is required but at the same time to be avoided due to bleaching.

Example: cells on a gel. DF will focus on coverslip, SWAF on cells. Choosing SWAF every 5 t-points, DF every t, limits phototoxicity.

• DF as reference for SWAF: speed up SWAF

Example: DF finds coverslip, distance to focus on cells is now minimal and can be performed much quicker by SWAF

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ZEISS, Sven Terclavers, Microscopy

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Focus Strategies Support Points

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Interpolate

Focus

Focus

• Create a Tile Region in Tiles window & select the tab Support Points to create positions that will be used as focus point to cover for sample tilting/tortuosity



Interpolate



Focus

Interpolate

Focus

Interpolate

Focus





Focus Strategies Support Points





• Why not SWAF Only, every nth Tile?

SWAF Only: every 3rd Tile Support Points: interpolation between points

Focus Strategies Focus Surface Support Points





- Uses Support Points (see earlier slide)
- **'By Tiles Setup**' does not run DF or SWAF, uses zposition of Positions or Regions as defined
- 'By SWAF' runs SWAF to define Support Points
- 'By DF: Find Surface' define position of coverslip, no further adjustment (works well on objectives with lower resolution due to long depth of focus)
- 'By DF: Find Surface + Additional Offset' applies an user-defined focus offset after DF finds the surface – similar to:
- **'By DF: Recall Focus'** uses the focus offset as defined by clicking Store Focus in the Definite Focus window

Focus Strategies Focus Surface Support Points with SWAF and DF combined

- 1. All Support Points, or Positions or Tile Regions (!) are first defined by DF
- 2. In a following step, SWAF will be performed based on settings in (3), as an 'Additional Action'
- 3. Similar settings to "Only SWAF or DF" (slides 12-14)





Focus Strategies Focus Surface Support Points with SWAF and DF combined

'Adapt Focus Surface/Z Values' has a few options:

For SWAF:

- 1. 'As Additional Action': see previous slide
- 2. **'Update with Single Offset**': only available with Time-lapse acquisition and will define a single offset on one position and apply this to all support points (faster, assuming cells are all on same distance from coverslip)

For DF:

- 1. 'As Additional Action': similar to SWAF
- 2. 'Update with Single Offset': similar to SWAF
- 'Update with Multiple Offsets': each position has a different offset. Covers situations where cells are moving combined with coverslip deformation due to e.g. heating stage. Distance between coverslip & sample is continuously variable.







When to choose which strategy?!







Software Autofocus:

- Preferably avoided for live cell imaging due to photo-bleaching/-toxicity, ok for tissue
- Requires some time to find focus
- Focus may be affected by dirt, bright spots

Definite Focus:

- Requires a refractive index mismatch between coverslip & medium to generate reflection
- Fast & accurate

Tiles Setup (fixed positions):

- Requires user input to define each single position
- More time-consuming

Choice of strategy

Cell culture: multiple position(s)/region(s), time-lapse





 Cells adhering to bottom

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- Cells not adhering to bottom
- Free floating in solution

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Choice of strategy

Cell culture: multiple position(s)/region(s), time-lapse







- Cells leveled, fixed, at homogeneous or heterogeneous thickness above coverslip
- No DF due to RI matching

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Disadvantage of 'By Tiles Setup' is that all z-positions have to be pre-defined

Choice of strategy

Cell culture: multiple position(s)/region(s), time-lapse





 Cells leveled, fixed, at heterogeneous thickness above coverslip
 DF finds coverslip

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- Cells leveled, fixed, at heterogeneous thickness above coverslip
- DF fails due to RI matching

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Choice of strategy Cell culture: time-lapse, long interval





- Cells adhering to bottom
- Single position

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- Cells adhering to bottom
- Multiple positions
- Cells growing on top of a gel or alike
- DF detects reflection
- Single/Multiple positions

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Alternatively, when adhering to coverslip, choose 'By Definite Focus: Find Surface'

Choice of strategy Tissue section: region(s), no time-lapse





- Small region
- About 3x3 tiles
- No support points



- Small, multiple
 regions
- About 3x3 tiles
- No support points

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- Large region(s)
- More than 3x3 tiles
- >9 support points

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Choice of strategy Local versus Global Support Points

- Instead of 'Local' Focus Support Points, 'Global' can be chosen. Global Support Points have to be defined in Sample Carrier Template
- Local Focus Points are always per position, or region
- Global Focus Points can compensate tilting of a large carrier at once, regardless of regions in each well
- Useful for:
 - Well-plate where cells are adhering to bottom (same fixed offset)
 - Outer wells can be filled with beads and used for SWAF without affecting sample in other wells







Focus Strategies with ZEN Summary



- A solution for every condition, it's a matter of finding the best option. Questions to ask:
 - Is the sample adhering? → include DF
 - Is the sample staying on a fixed distance of the bottom? → **DF with Recall Focus**
 - Is DF not finding a reflection? → include By Tiles Setup (or SWAF)
 - Floating/tortuous? → include SWAF
 - Time-lapse with long interval? → remember to Enable Periodic Stabilization
- Be creative:
 - If SWAF is required, consider using far-red beads if possible \rightarrow no effect on other channels
 - Consider using Global Support Points where some wells only contain beads
 - Try SWAF on brightfield channel
- But above all: make sure the system's temperature is stabilized. A system that is warming up will show focus maintenance instability.

